Redox Potential-Controlled 1,3-Propanediol Production from Glycerol by *Lactobacillus panis* PM1

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Abstract

1,3-propanediol is a bulk chemical that has been mostly used as a monomer in the synthesis of fiber polymer. The biobased 1,3-propanediol has widely applications in food as sweetener, cosmetics as moisturizer, and pharmaceutical industry as solvent. *Lactobacillus panis* PM1, originally isolated from thin stillage coming from a local bioethanol fermentation plant, is evaluated as a potential organism to produce 1,3-propanediol from both glucose and glycerol. *Lactobacillus panis* PM1 strain is an aerotolerant acidophilic anaerobe, which can grow over a wide range of temperature; tolerant to high ethanol, acetate and lactate concentrations; and resistant to many common antibiotics. In this study, the strain was cultivated under various fermentation redox potential-controlled environments, attempting to evaluate the effectiveness of fermentation redox potential control on 1,3-propanediol production. Results had shown that fermentation redox potential regulation could significantly improve fermentation efficiency in terms of reduced fermentation time and improved 1,3-propanediol production. The addition of potassium ferricyanide as oxidant, could significantly accelerate glycerol utilization rate along with 1,3-propanediol production. The best fermentation redox potential level was -200 mV, in which fermentation time had been reduced into half from 104 h to 49 h, 1,3-propanediol production was improved by 24.2% to 23.38 g/L ,while productivity was raised 182% to 0.48 g/L-h. The most optimized initial molar ratio of glucose to glycerol was also evaluated under fermentation redox potential controlled at -200 mV. The initial molar ratio of glucose to glycerol between 0.30-0.40 gave the highest fermentation efficiency among all the investigated conditions.

Acknowledgements

I would like to express my sincere gratitude to my supervisors, Dr. Yen-Han Lin, who has been always supportive, encouraging and patient. He has guided and inspired me though the whole project. I am grateful to my committee, Dr. Takuji Tanaka and Dr. Venkatesh Meda for their precious academic advices for my project. Special appreciation for Dr. Tanaka, who provided the strains, allowed me to use his lab and guided me through all aspects of cultivating recombinant strain. I want to thank all my friends, especially for Xi Xie and Kaiwen Sun, for their friendship, valuable discussions, and encouragement throughout my graduate research.

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List of Abbreviations

ADH	Alcohol Dehydrogenase
AK	Acetate Kinase
ALDH	Acetaldehyde Dehydrogenase
ATP	Adenosine Triphosphate
CL	Citrate Lyase
DO	Dissolved Oxygen Level
EM	Embden-Meyerhof Pathway
ENO	Enolase
FH	Fumarate Hydratase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDHt	Glycerol Dehydratase
GLK	Glucokinase
G6PDH	Glucose 6-Phosphate Dehydrogenase
3-HPA	3-Hydroxypropionaldehyde
LDH	Lactate Dehydrogenase
MDH	Malate Dehydrogenase
ME	Malic Enzyme
NAD^+/NADH	Nicotinamide Adenine Dinucleotide
ORP	Fermentation Redox Potential
PDH	Pyruvate Dehydrogenase
PDO	1,3-Propanediol
PDOR	1,3-Propanediol Dehydrogenase
6-PG/PK	6-Phosphogluconate/Phosphoketolase
PGDH	6-Phosphogluconate Dehydrogenase
PGK	Phosphoglycerate Kinase
PGM	Phosphoglyceromutase
PTT	Polytrimethylene terephthalate
PK	Pyruvate Kinase
PM1	*Lactobacillus panis* PM1
PTA	Phosphotransacetylase
PTT	Polytrimethylene Terephthalate
R	Initial Molar Ratio of Glucose to Glycerol
RPE	Ribulose 5-Phosphate 3-Epimerase

SDH	Succinate Dehydrogenase
OD	Optical Density
XFP	Xylulose 5-Phosphate Phosphoketolase

1

Introduction

1,3-Propanediol (PDO) is one of the most extensively used monomers in synthesizing polyesters and heterocyclic compounds. It also has been widely applied in the food, cosmetic and pharmaceutical industries[1]. The global market of PDO is expected to reach $621.2 million by 2021 at compound annual growthrate of 10.4% from Markets and Markets 2012 report [2]. Since the chemical synthesis of PDO is highly unsustainable, the development of bioconversion from renewable resources has been gaining more importance [3]-[4].

Klebsiella sp. and *Clostridium sp.* are the most studied PDO producing strains due to their substrate tolerances, high yields, and productivities. However, *Klebsiella sp.* is opportunistic pathogen and *Clostridium sp.* is strict anaerobic [1],[3]-[4]. *Lactobacillus*, known as "generally recognized as safe", has been industrialized in food and agriculture fermentation for centuries. Products driven from *Lactobacillus* can also be safely used in cosmetic and pharmaceutical industries. Recently, bioconversion of PDO from glycerol has been paid more attention due to sustainability and low cost of crude glycerol. Glycerol can be converted into PDO with the presence of glucose by heterofermentative *Lactobacillus* through reductive pathway under anaerobic or micro-aerobic fermentation at 37℃[5].

Lactobacillus panis PM1 (PM1), is a natural PDO producer isolated from bioethanol plant thin stillage. PM1 belongs to heterofermentative *Lactobacillus*, which metabolizes glucose via 6-phosphogluconate/ phosphoketolase (6-PG/PK) pathway. Glucose is metabolized as the primary energy source to yield lactate, acetate, and ethanol [6]. However, since the low acetaldehyde dehydrogenase and alcohol dehydrogenase activities, ethanol production becomes the growth limiting step. To overcome the problem, heterofermentative *Lactobacillus* utilizes external electron acceptors, such as glycerol, oxygen and citrate, to maintain redox balance and to assist cell growth [7]-[8]. In the case of PDO fermentation, glycerol is metabolized through an additional reductive pathway by two key enzymes: glycerol dehydratase and 1,3-propanediol dehydrogenase. The presence of glycerol shifts the ethanol production to acetate and PDO production. The theoretical yield of glycerol to PDO is 1 mol/mol with glucose as co-substrate. Without glucose, the yield of PDO on glycerol is 0.85 mol/mol under micro aerobic condition [9]. The highest PDO production achieved by PM1 was 100.78 mM (7.67 g/L) from 56 mM (10.00 g/L) glucose and 150 mM (13.80 g/L) glycerol [10]-[11].

Redox potential-controlled fermentation can alter metabolic profiles and improve fermentation efficiency. During very-high-gravity ethanol fermentation, the fermentation time had been reduced, and ethanol fermen-

tation efficiency by *S. cerevisiae* was the highest when ORP controlled at -150 mV was regulated by sparing air into fermenter [12]. In PDO fermentation by *K. pneumoniae*, the highest PDO productivity was achieved under ORP regulated at -190 mV and -160 mV by air sparing [13]. ORP regulation can also be applied in recombinant strain fermentation, where ORP controlled at -400 mV had improved succinate production by recombinant *E. coli* [14]. In this study, we attempted to incorporate ORP regulation to PM1 to produce PDO from glycerol with co-fermentation of glucose. Being an anaerobic strain, the common air or mixed air-nitrogen sparging techniques to control ORP were ineffective. Alternatively, the use of chemical-based oxidant was chosen. Potassium ferricyanide, an inorganic-based oxidant, features a high reduction potential (436 mV) was used in this study. The following ORP levels were implemented: no control, controlled at -250, -200, and -150 mV, respectively, to investigate the effect on PDO production, substrate utilization and the overall fermentation efficiency.

1.1 Project Motivation and Knowledge Gap

Several fermentation strategies, including batch, repeat-batch, two-stage fermentation, continuous and fed batch fermentation with suspended cells and immobilized cells, are utilized for PDO production. Microbial strains genera involved in PDO production include *Klebsiella*, *Clostridium*, *Enterobacter*, *Citrobacter* and *Lactobacillus* [1]-[4],[15]. Whereas the otherse opportunistic pathogenes or strict anaerobic strains, *Lactobacillus* is generally recognized as safe, and can grow in the presence of oxygen, i.e., it is a facultative anaearobe. The strain is recently proposed as a potent industrial strain as PDO producer. Heterofermentative *Lactobacillus* utilizes glycerol, as external electron accepter, in glucose anaerobic fermentation to either enhance or maintain the growth. It shifts the metabolic flux from lactate and ethanol production to acetate and PDO, yielding one extra mole of ATP and re-oxidizing NADH [5],[8]. *L. reuteri* and *L. diolivorans* are most studied heterofermentative *Lactobacilli* PDO producing strains, *L. diolivorans* DSM14421, could produce up to 92 g/L PDO in fed-batch [16], and *L. reuteri* DSM20016 could produce 46 g/L in batch fermentation [17]. According to previous study, PM1 could produce up to 100.78 mM (7.67 g/L) PDO in batch fermetnation [10]. The experiment was conduced in lab scale and the experimental parameters had not been optimized to improve PDO production in fermentor. Since *L. reuteri* and *L. diolivorans* have similar metabolic pathway compared with PM1, the experimental parameters of those two strains can be applied as reference to optimize PM1 fermentation. In this study, the fermentation was conduced at 37℃, pH 5.5 with agitation of 100 rpm.

PDO production depends on the initial glucose and glycerol concentration. Glucose is the preliminary energy source for cell propagation, and it provides reducing equivalents during glycolysis for glycerol reduction to produce PDO. The final PDO production is directly correlated with glycerol but is also negatively affected when glycerol concentration is high. The presence of glycerol in high amount can reduce the permeability of the cell membrane, which limits nutrient entrance [5],[18]. In batch fermentation, PM1 can survive up to 870 mM (80.1 g/L) glycerol, while PDO production is inhibited up to 300 mM (27.6 g/L). High glycerol

concentration also suppresses the activities of two genes: glycerol dehydratase and 1,3-propanediol dehydrogenase related with glycerol reduction. The optimal molar ratio of glucose to glycerol for PM1 is 0.30-0.40 [6],[10]-[11].

1.2 Hypothesis

Among those heterofermentative *Lactobacilli* fermentation, pH, temperature, agitation, aeration and substrates concentrations were investigated to optimize the batch fermentation in PDO production. Facultative to strictly anaerobic conditions were required during fermentation, the batch fermentation time varied from 48 h to 249 h, and the final PDO concentration was between 7.8-46 g/L [10],[17]. It has been demonstrated that fermentation redox potential (ORP) was effective to monitor and control *K. pneumoniae* fermentation in PDO production. ORP-controlled at different levels was correlated with various NAD^+/NADH ratio, in which -190 mV was the most favorable condition for PDO production [13]. Since PDO production is dependent on glycerol reduction and redox balance, in addition to all the defined experimental parameters from previous studies, an ORP-controlled fermentation would improve PDO production while reduce fermentation time by shifting the metabolic pathway.

1.3 Research Objectives

PM1 was grown under ORP-controlled environment to evaluate it as a potential organism to produce PDO from both glucose and glycerol. The previous results had shown that this strain could utilize glucose to initiate the cell growth. After the depletion of glucose, a diauxic shift was observed where glycerol became the sole carbon source and the production of PDO increased [7]-[10]. The above-noted observation and results are promising that the strain could produce PDO; however, the fermentation conditions need to be optimized to enhance fermentation efficiency and PDO productivity.

We proposed to investigate the following combinations of fermentation conditions and operations:

1. Control fermentation under anaerobic condition by controlling fermentation redox potential. According to literature survey, I would tentatively adopt the redox potential at -200±50 mV to begin the fermentation. As fermentation proceeds, I would vary ORP range to improve the fermentation performance accordingly.

2. Optimization of initial concentrations of glucose and glycerol. Glucose builds up the cell population, while glycerol is reduced to PDO. According to the literature review, the highest PDO production from PM1 occurs at the initial molar ratio of glucose to glycerol (R) between 0.30-0.40. As experiments proceed, this initial concentration of the substrates would be adjusted accordingly to minimize substrates inhibition.

1.4 Organization of Thesis

The work presented in this thesis resulted in one manuscript. The thesis is written in the manuscript-based style and organized into four chapters. The introduction is presented in Chapter 1, along with project motivation and knowledge gap; hypothesis; research objectives; and organization of thesis. In Chapter 2, the literature review provides some background in PDO applications and productions; glucose and glycerol metabolism in *Lactobacillus*; strain PM1; and ORP-controlled fermentation. Chapter 3 includes the manuscript with focus on ORP-controlled PDO production by PM1. Finally, a summary of results; conclusions; and recommendations and future work are presented in Chapter 4. In this work, the reference is provided at the end of this thesis, followed by appendices where experimental setup, PM1 inoculation conditions and permission to use forms are provided.

2

Literature Review

In the following sections, background information regarding with the targeting product 1,3-Propanediol(PDO)'s applications and manufacture methods; glucose and glycerol metabolism in *Lactobacillus*; the strain *Lactobacillus panis* PM1; and redox potential-controlled fermentation are provided.

2.1 1,3-Propanediol Application and Production

1,3-propanediol (PDO) is an organic compound with formula $C_3H_8O_2$. PDO is colorless, non-flammable, low toxicity, viscous liquid which is miscible with water and ethanol [3]. It is a valuable chemical that has been applied in many industries shown in Figure 2.1 [1].

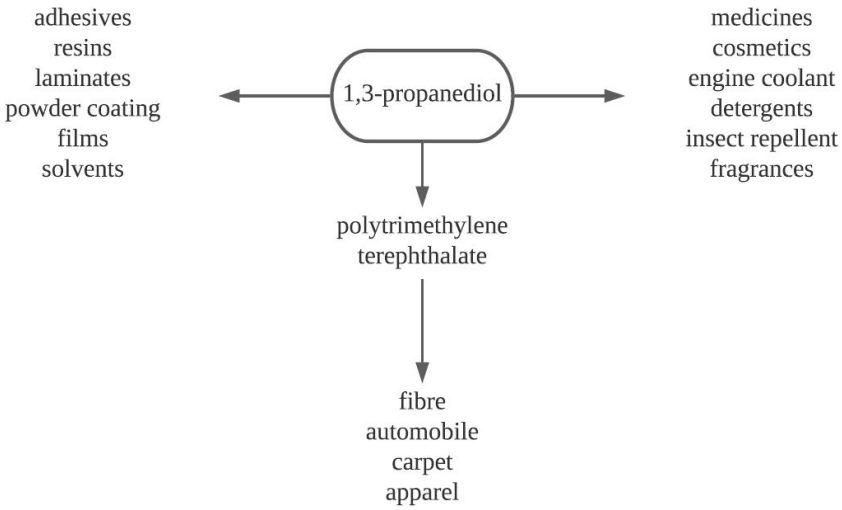


Figure 2.1: Industry Application of PDO [1]

The global market of PDO is expected to reach $621.2 million by 2021 at compound annual growth rate of 10.4% [2]. PDO is an important chemical intermediate in manufacturing of polyethers, polyurethanies, biocides and heterocyclic compounds. The majority of PDO has been utilized to synthesis polytrimethylene terephthalate (PTT), a biodegradable polymer used in carpet, textile, film, and packaging industry [1]. The PPT driven from PDO has a coil-like or zig-zag shape, leading to better stretch-recovery property, lower dyeing temperature, higher UV resistance and better wash fitness than other commercially available

polyesters [1],[3]. Shell Chemicals and DuPont have been manufacturing PTT through polycondensation of terephthalic acid or dimethyl terephthalate with PDO. PTT is synthesized by the PDO transesterification with dimethylene terephthalate or PDO esterification with terephthalic acid. The reaction is carried out with metal catalyst, such as titanium butoxide and dibutyl tin oxide, at 260℃ with acrolien and allyl alcohol as main byproducts [19].

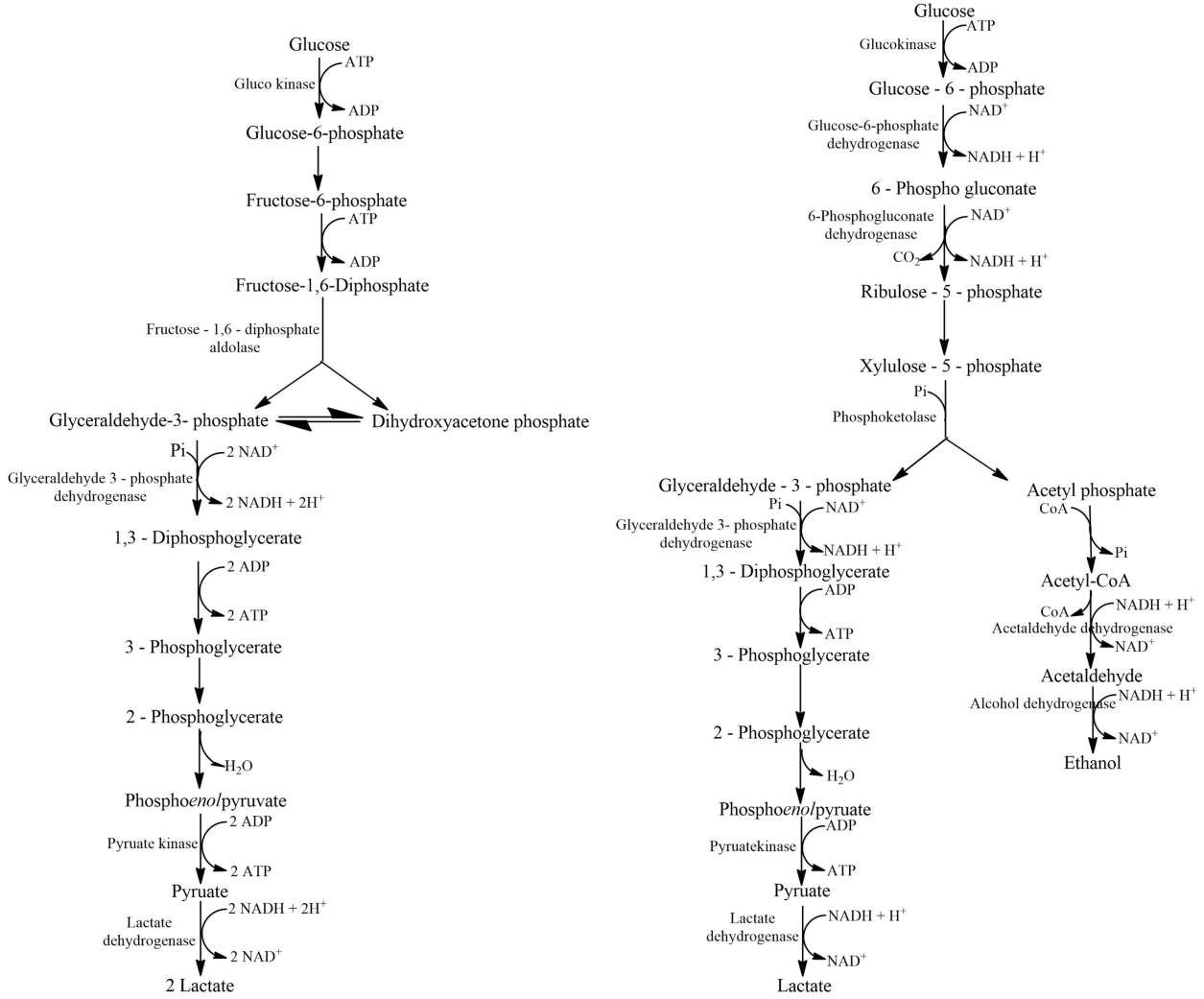
The current PDO market is mainly shared among Dupont, Shell and DuPont Tate & Lyle. Dupont and Shell Chemicals produce PDO, through catalytic chemical process from crude oil-based raw material, by hydration of acrolein and hydroformylation of ethylene oxide. These two processes give yield of 40% and 80%, respectively. The chemical process requires high pressure and temperature, and expensive, non-renewable petroleum-based catalysts, while it also releases toxic intermediates. As far as the environment and sustainability concerned, the bioconversion of PDO which is particularly driven from renewable feedstock and cultivated at normal temperature and pressure leading to no generation of toxic by-products, has been gaining prominence. Currently, DuPont Tate and Lyle has developed PDO synthesis from corn syrup by recombinant *E. coli* [1],[3],[15].

2.2 Glucose and Glycerol Metabolism in *Lactobacillus*

Several *Lactobacilli* are generally recognized as safe and have been industrially utilized in food and agriculture fermentations. *Lactobacillus* lacks of respiratory system and utilizes substrate level phosphorylation as energy source. The strains have been classified into three main groups based on fermentative characteristics and metabolic profiles. Group I obligatory homofermentative *Lactobacillus* can metabolize hexose into lactate as the only product through Embden-Meyerhof (EM) pathway. As shown in Figure 2.2a [5], one mole glucose is metabolized into two moles pyruvates, while yielding two moles ATP and NAD^+. Pyruvates are further reduced into two moles lactate. Since pentose and gluconate cannot be metabolized, no carbon dioxide is generated in this type of fermentation. Group I *Lactobacillus*, including *L. acidophilus, L. delbrueckii, L. helveticus and L. saluvarius*, are commonly utilized in cheese and bread fermentation [5],[10].

Group II facultative heterofermentative *Lactobacillus* ferments hexose and pentose into lactate and/or acetate by EM pathway, while producing carbon dioxide from gluconate. *L. casei, L. plantarum*, and *L. sakei* are typical Group II *Lactobacilli* used in meat and vegetable fermentation. Group III obligatory heterofermentative *Lactobacillus* can metabolize hexose and pentose into lactate, ethanol and/or acetate and carbon dioxide through 6-PG/PK pathway [19]. *L. reuteri* is one of the most well-studied Group III *Lactobacillus* used as probiotic. Other few strains are responsible for sourdough fermentation, but most are involved in food spoilage [5],[10]-[11],. The metabolism for heterofermentative *Lactobacillus* is shown in Figure 2.2b [5].

However, hexose fermentation negatively affects the cell growth in heterofermentative *Lactobacillus*. The 6-PG/PK pathway is optimized for pentose fermentation. In early oxidation stage, hexose requires one



(a) Glucose Metabolism in Homofermentative *Lactobacillus*

(b) Glucose Metabolism in Heterofermentative *Lactobacillus*

Figure 2.2: Glucose Metabolism in *Lactobacillus* [5]

extra mole ATP by glucokinase to convert into glucose-6-phosphate. Two extra moles NADH are reduced from hexose to pentose conversion. Those NADH cannot been efficiently oxidized via ethanol production from acetyl-phosphate/CoA, due to the low acetaldehyde dehydrogenase activity. To overcome the problem, heterofermentative *Lactobacillus* applies external electron acceptors, such as citrate, glycerol and oxygen, to re-oxidase NADH in addition to ethanol pathway [8].

Glycerol dissimilation is coupled with oxidation and reduction reaction. Glycerol reduction produces PDO, while regenerating the reduced NAD^+ for pentose phosphate pathway to maintain the redox balance. The transportation of glycerol into bacteria can be achieved by both passive diffusion at high glycerol concentration,and glycerol facilitator (GlpF), an integral membrane protein, at low concentration. *Lactobacillus* cannot metabolize glycerol through oxidative pathway due the lack of *glp* regulon and respiration pathway. It requires hexose through substrate level phosphorylation as its primary metabolism, while glycerol is metabolized through additional reductive pathway to re-oxidize NADH and to maintain redox balance [8]. The present of glycerol enhances the growth of *Lactobacillus* by shifting the products from lactate and ethanol into acetate and PDO with one extra mole of ATP produced from acetate.

The genes responsible for glycerol metabolism in *Lactobacillus* are encoded in *pdu* operon. *pdu* enzyme is responsible for glycerol dissimilation and microcompartment formation. There are two key enzymes involved to catalyze PDO production in glycerol reductive pathway as shown in Figure 2.3 [20]: glycerol dehydratase (GDHt), and 1,3-propanediol dehydrogenase (PDOR). GDHt is class II coenzyme B_{12}-dependent enzyme encoded by *dhaB*. It converts glycerol to 3-hydroxypropanaldehyde (3-HPA). Both PM1 and *L. reuteri* are natural Vitamin-B_{12} producers [10], where the reaction happens in micro-compartment inside the cytoplasm. A regulatory protein *poc* R, encoded in the upperstream of Vitamin B_{12} synthesis and *pdu* operon, is found in *L. reuteri* for PDO and Vitamin B_{12} synthesis. The overexpression study of this protein has proved that it could shift the metabolic flux from ethanol into PDO and acetate [21]. However, the intermedia, 3-HPA, is toxic to microorganism, which inhibits PDO production. PDOR is NADH dependent enzyme encoded by *dhaT*. It directly hydrogenates 3-HPA to PDO while regenerating reducing equivalent. The presence of metal ions, such as Mn^{2+} and Fe^{2+} positively affects PDOR activity [18],[20],[22]. The theoretical yield of glycerol to PDO is 1 mol/mol with glucose as co-substrate. Without glucose as co-substrate, the yield of PDO on glycerol is 0.85 mol/mol under micro aerobic condition [9].

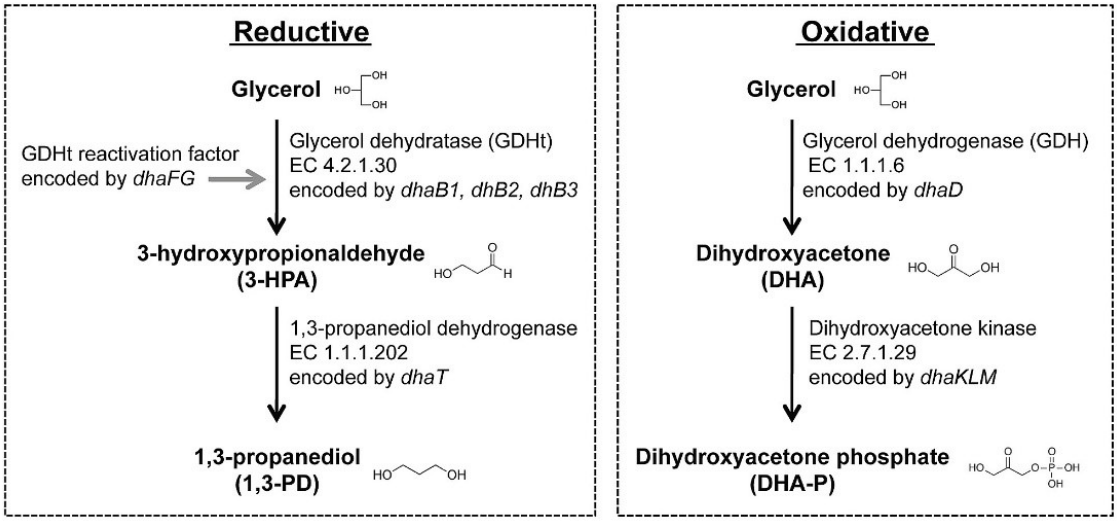


Figure 2.3: Glycerol Metabolism in Reductive Pathway [20]

2.3 *Lactobacillus panis* PM1

Lactobacillus panis PM1 (PM1) can metabolize glycerol into value-added chemical PDO. PM1 belongs to Group III heterofermentative *Lactobacillus* that utilizes glucose via 6-PG/PK pathway as its primary metabolism [6]. The metabolic behavior of PM1 is corresponded with the other two heterofermentative PDO producing *Lactobacilli*: *L. reutri* and *L. diolivorans*. As shown in Figure 2.4 [7], PM1 first metabolizes one mole hexose into one mole pentose plus one mole carbon dioxide, then splits pentose into equal molar of pyruvate and acetyl-phosphate. Meanwhile, three moles NADH and one mole ATP are generated. NADH oxidation is achieved via the reduction of pyruvate to lactate and acetyl-phosphate to ethanol to maintain redox balance. One additional mole of ATP is generated via acetate production from acetyl-phosphate. Ethanol production is the cell growth limiting step since low acetaldehyde dehydrogenase and alcohol dehydrogenase activities inhibit NADH oxidation. Under the presence of glycerol as external electron acceptor, the NADH oxidation route shifts from ethanol to PDO production. Most acetyl-phosphate is converted by acetate kinase to acetate to yield extra mole of ATP rather than ethanol. Pyruvate oxidase converts part of available pyruvate into acetyl-phosphate/CoA instead of lactate, and release one mole carbon dioxide. One extra mole ATP is produced via acetate production. After glucose exhaustion, lactate dehydrogenase re-oxidizes accumulated lactate back to pyruvate, then to acetyl-phosphate and acetate. PM1 also metabolizes citrate to re-direct NADH oxidation to succinate production [7]-[11].

PDO production and glycerol reduction is dependent on NADH accumulation from glucose metabolism, Higher initial glucose concentration positively affects the glycerol reduction leading to higher PDO production. PDO production also depends on initial glycerol concentration, temperature, and pH. The best growth of PM1 is observed under anaerobic condition at 37℃, pH 4.2 in MRS medium with glucose, glycerol and citrate, yielding succinic acid, acetate, lactate, PDO and ethanol. The highest PDO production is 100.78 mM (7.67

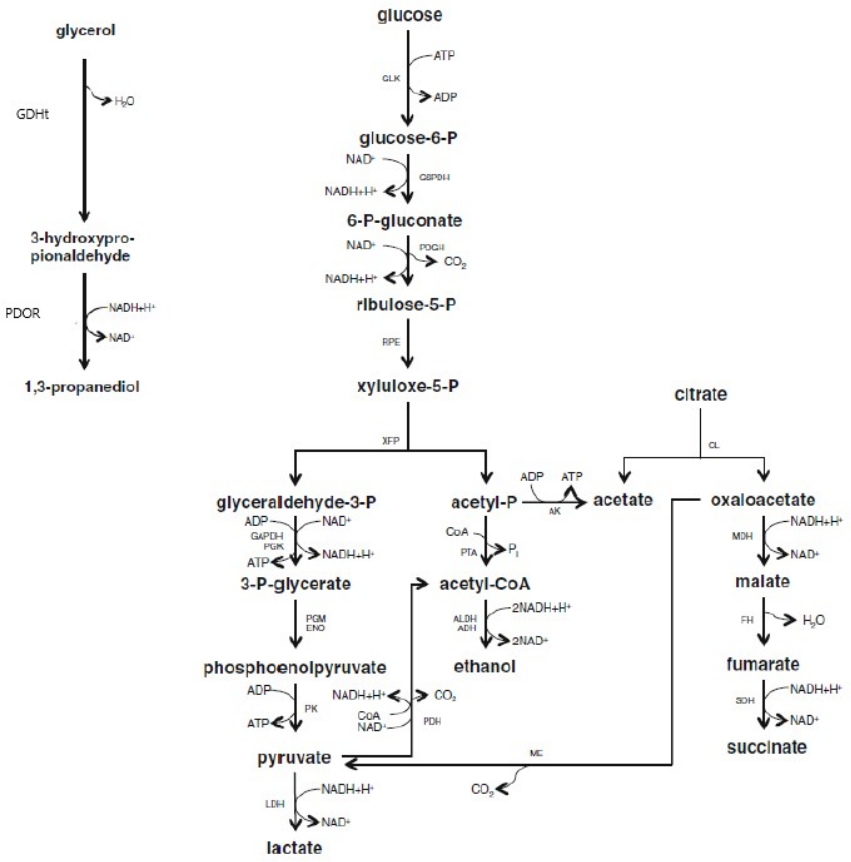


Figure 2.4: Glucose and Glycerol Metabolism in *Lactobacillus panis* PM1 [7]

g/L) with 56 mM (10.0 g/L) glucose and 150 mM (13.8 g/L) glycerol [10]-[11].

2.4 Redox Potential-Controlled Fermentation

Fermentation refers to pyruvate metabolism in the absent of oxygen. Pyruvate is reduced into different metabolites via NAD(P)H oxidation to $NAD(P)^+$ as shown in Figure 2.5 [23]. $NAD(P)^+$ and NAD(P)H are the most essential electron carriers contributed to intercellular redox potential. In catabolism, NADH donates one electron for matebolites formation, whereas NADPH is involved in amino acids, fatty acids and nucleic acid production in anabolism. Intercellular redox potential is closely related with oxidative phosphorylation, which strains utilize redox potential sensitive enzymes to oxidize nutrients, and to release energy stored for ATP production. Since bacteria do not possess mitochondria, the reactions occur across the plasma membrane. Electrons are transfered through series of oxidation and reduction reactions catalyzed by electron transport chain. Oxygen, the terminal electron acceptor, takes the electron to form water. The permeable plasma membrane selects external redox chemicals to enter the cytoplasm. Therefore the intercellular redox potential changes. Oxygen and dithiothreitol can pass the membrance freely. Others, such as ferric reductase, require the assitance of membraine proteins, oxidoreductase, located on electron transport chain [24]-[25].

The intracellular microorganism activities and redox potential, including energy and material metabolism, can be reflected by external redox potential level. Fermentation redox potential (ORP) is the net balance of oxidation and reduction within microorganism, measured in voltage difference (mV). ORP is correlated with fermentation pH, temperature, dissolved oxygen level (DO) and agitation. Since enzymes related to oxidation and reduction are redox potential sensentive, ORP regulation can re-direct intracellular metabolic flux to desired metabolites formation, leading to higher fermentation efficiency [23]. During very-high-gravity ethanol fermentation, the fermentation time had been reduced, and ethanol fermentation efficiency by *S. cerevisiae* was the highest at ORP controlled at -150 mV regulated by sparing air into fermenter [12]. In PDO fermentation by *K. pneumoniae*, the highest PDO productivity was achieved under ORP regulated at -190 mV and -160 mV by air sparing [13]. ORP regulation can also be applied in recombinant strain fermentation, where ORP controlled at -400 mV had improved succinate acid production by recombinant *E. coli* [14].

ORP regulation can be achieved by controlling the amount of terminal electron acceptor and donor added to fermentation environment. For aerobic strain, air or mixed air-nitrogen is the most feasible method for ORP regulation to increase the ORP level. Oxygen is a strong terminal electron acceptor who picks up the electrons passed by electron transport chain. It barely has inhibition effect on strain growth. For anaerobic strain, potassium ferricyanide and dithiothreitol are commonly used pair of oxidant and reductant [23]. ORP trends to be more sensitive under microaerobic and anerobic conditions, since oxygen trends to take electrons even its concentration is lower than others. The ferric ion presented in potassium ferricyanide

receives electrons to form ferrous ion in order to raise ORP [26].

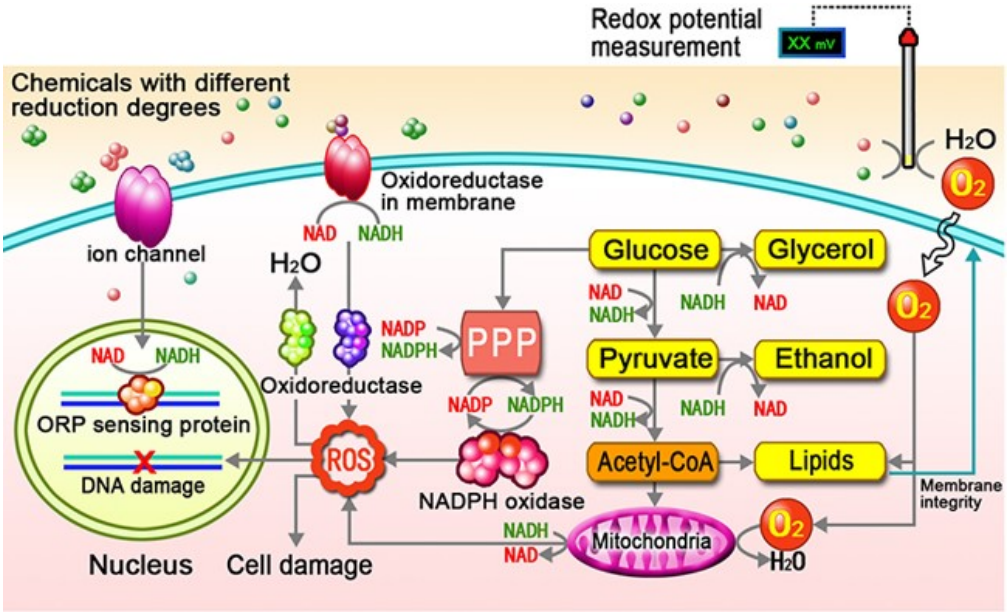


Figure 2.5: Reduction and Intracellular Redox Response to Extracellular Redox Potential and Effects of Redox Potential on Cellular Metabolism and Stress Response [23]

3

Redox Potential-Controlled 1,3-Propanediol Fermentation from Glycerol by *Lactobacillus panis* PM1

Contribution of the MSc Student

Experiments were planned and performed by Vicky Wu with the guidance provided by Dr. Lin, who also provided consultation during the entire experimental period as well as thesis preparation. *Lactobacillus panis* PM1 (PM1) was provided by Dr. Tanaka. All the writing of the manuscript was done by Vicky Wu with Dr. Lin, providing editorial guidance regarding the style and content of the paper.

Contribution of this Chapter to the Overall Study

In this chapter, the optimal redox potential (ORP) regulation level for 1,3-Propanediol (PDO) fermentation by PM1 was determined by conducting ORP-controlled fermentation at -250, -200 and -150 mV and without control. The pH,temperature and agitation for all fermentation were kept constant. When ORP was controlled at -200 mV, the PDO production and productivity was the highest (23.38 g/L and 0.48 g/L/h). In addition, the most optimal ratio between glucose to glycerol when ORP was controlled at -200 mV, in terms of highest PDO production, was 0.30 to 0.40.

3.1 Abstract

The fermentation redox potential (ORP) was controlled to investigate the fermentation efficiency during the production of 1,3-propanodiol (PDO) by *Lactobacillus panis* PM1 (PM1). Results show that the best redox potential level was -200 mV at which had the highest PDO production (23.38 g/L) when compared to control at -250 mV and -150 mV, and without control. For the ORP levels under investigation, the mass yield (with respect to glycerol consumption) was 0.66-0.82. Potassium ferricyanide was used as an oxidant to control ORP level. With the addition of oxidant, the batch fermentation time was noticeably reduced compared to the one without ORP control. Furthermore, co-substrate utilization of glucose and glycerol was observed when potassium ferricyanide was presented. It was postulated that such co-substrate utilization pattern was resulting from the redox imbalance where the activities of acetaldehyde dehydrogenase and alcohol dehydrogenase were retarded by the presence of potassium ferricyanide. To overcome the redox imbalance, the glycerol-reductive pathway was trigged to serve as the electron source to fuel glycolysis pathway.

3.2 Introduction

With the worsening of global warming, the green processing of bulk chemicals becomes attractive. Many of bulk chemical are made with the involvement of fossil fuel. The use of fermentation technology for bulk chemicals production is a potential alterative [1],[3]. 1,3-propandiol (PDO) is an important polyvinyl chloride intermediate, and is made from fossil fuel. Thus alternative fermentation production would be benificial. Several fermentation strategies, including batch, repeat-batch, two-stage fermentation, continuous and fed-batch fermentation with suspended cells and immobilized cells, are utilized for PDO production [4]. Microbial strains genera involved in PDO production include *Klebsiella, Clostridium, Enterobacter, Citrobacter* and *Lactobacillus*. Among those strains, facultative to strictly anaerobic conditions are required during fermentation, the batch fermentation time varied from 48 h to 249 h, and the final PDO concentration was between 7.8-46 g/L [1],[3]-[4]. Whereas many of these strains are opportunistic pathogenic strains, *Lactobacillus* is generally recognized as safe; therefore it is a very attaractive option to produce PDO [5]. *L. diolivorans* and *L. reuteri* are two most studied PDO producing *Latobacilli* strains: *L. diolivorans* DSM14421, could produce up to 92 g/L PDO in fed-batch [16], and *L. reuteri* DSM20016 could produce 46 g/L in batch fermentation [17].

It has been demonstrated that fermentation redox potential (ORP) could alter metabolic profiles and improve fermentation efficiency. During very-high-gravity ethanol fermentation, the fermentation time had been reduced, and ethanol fermentation efficiency by *S. cerevisiae* maintenance of ORP at -150 mV by air sparging fermentation achieves the highest [12]. In PDO fermentation with *K. pneumoniae*, the highest PDO productivity was achieved under ORP regulated at -190 mV and -160 mV by air sparging [13]. ORP regulation can also be applied in recombinant strain fermentation, where ORP controlled at -400 mV had improved succinate production by recombinant *E. coli* [14].

In this study, we attempted to incorporate ORP-control to PM1, isolated from a local bioethanol fermentation plant, to convert glycerol into PDO with co-fermentation of glucose [6]. In previous study, this strain was observed to produce 7.67 g/L PDO from 10.0 g/L glucose and 13.8 g/L glycerol [10]. Being a facultative anaerobic strain, the common air or mixed air-nitrogen sparging techniques to control ORP were ineffective. Alternatively, the use of chemical-based oxidant was chosen. The following ORP level were implemented: no control, controlled at -250, -200, and -150 mV, respectively, to investigate its effect on PDO production, substrate utilization and the overall fermentation efficiency.

3.3 Methods and Materials

3.3.1 Strain and Media

All the chemicals used in this study were purchasd from Fisher Scientific (Ottawa, ON, Canada).

Lactobacillus panis PM1 was kindly provided by Dr. Takuji Tanaka [1]. The modified MRS media

contained (per liter) 21.5 g glucose, 5 g yeast extract, 10 g peptone, 10 g meat extract, 2 g K_2HPO_4, 2 g ammonia citrate, 5 g sodium acetate, 100 mg $MgSO_4 \cdot 7H_2O$, 50 mg $MnSO_4$ and 33 g glycerol.

3.3.2 Fermentation and Redox Potential Measurement and Regulation

The fermentation was conducted in a 2-L fermentation jar (Virtis, SP Industries, Warminster, PA, USA) with 1.8-L defined culture. The start culture, 300-mL, was incubated at 37℃ under static condition until optical density (OD_{600}) reached 1 for inoculation. Agitation rate was maintained at 100 rpm and pH was regulated by 2N NaOH at 5.5. During fermentation, pH, ORP, dissolved oxygen (DO) and temperature were monitored by ORP and DO electrodes (ORP: Mettler Toledo Inc. Pt 4805-DPAS-SC-K8S; DO: InPro6800, Billerica, MA, USA). ORP level was regulated by potassium ferricyanide as oxidizing agent to raise ORP to a pre-determined level. After ORP level had dropped below -100 mV, a 10-mL sample was withdrawn periodically to evaluate OD_{600} and metabolite profile. The substrate utilization and product production rates were calculated based on the change of concentrations from initial amounts with respect to time.

3.3.3 Analytical Procedures

Biomass concentration was determined based on OD_{600} by UV-VIS Spectrophotometer (Shimadzu UV1800, Kyoto, Japan) after proper dilution and dry cell weight (DCW) obtained after samples had been centrifuged at 10,000 rpm for 10 mins. The correlation between DCW (g/L) and OD_{600} of PM1 culture was evaluated as DCW=0.266 OD_{600}+0.089. The metabolite profile, including glucose, lactate, glycerol, acetate, PDO and ethanol, was determined by HPLC (Agilent 1260 series, Agilent Technologies, Mississauga, ON, Canada), coupled with RI director (Agilent 1260 series, Agilent Technologies, Mississauga, ON, Canada) and IC Sep column (ION-300 300*7.8 mm, Transgenomic Inc., Omaha, NE, USA). The column was operated at 70℃ with 8.5 mM H_2SO_4 at 0.4 mL/min.

3.4 Results and Discussion

3.4.1 Metabolite Profiles of *Lactobacillus panis* PM1 without Redox Potential Control

In this study, the metabolism of PM1 was observed during batch cultivations carried out in modified MRS medium with 119.22 mM (21.5 g/L) glucose and 358.10 mM (33.0 g/L) glycerol under different ORP regulations to examine PDO productivity. ORP and biomass (expressed by OD_{600}) profiles under various ORP regulation are illustrated in Figure 3.1. The corresponding metabolite profiles of glucose, glycerol, lactate, PDO, acetate and ethanol are presented in Figure 3.2. Figure 3.3 illustrates the glucose and glycerol consumption rates and lactate and PDO production rates with different ORP regulations. Table 3.1 summarizes

fermentation results obtained under all experimental conditions. Table 3.2 compares PDO productivity obtained in the current study to literature data.

As reported from previous study, PM1 mainly utilizes glucose for propagation along with the production of lactate, acetate, and ethanol. After the depletion of glucose, a switch to the utilization of glycerol is observed, where PDO is synthesized along with the consumption of both glycerol and lactate [7]-[10],[8]. The metabolic behavior of PM1 is corresponded with the other two reported Group III heterofermentative *Lactobacilli*, *L. diolivorans* and *L. reuteri*. These heterofermentative *Lactobacilli* utilize glucose via 6-phosphogluconate/phosphoketolase pathway for propagation. The central metabolism and the reductive glycerol pathway for PM1 has been previously illustrated and shown in Figure 2.4 [7]. This strain metabolizes one mole of glucose into one mole of each pyruvate and acetyl-phosphate, while generating a net of one mole of ATP and three moles of NADH. NADH oxidation is achieved via the reduction of pyruvate to lactate and acetyl-phosphate to ethanol. However, the ethanol production is the growth-limiting step of heterofermentative *Lactobacillus* since low acetaldehyde dehydrogenase and alcohol dehydrogenase activities cannot completely re-oxidize the extra two moles of NADH generating during glycolysis, resulting in redox imbalance. To overcome the problem, heterofermentative *Lactobacillus* requires the addition of external electron acceptors, such as citrate, glycerol, or oxygen [8],[10],[27]-[28].

In this study, extracellular ORP, as an indicator of microbial activity, had been monitored throughout the fermentation process. ORP was correlated with OD_{600} and metabolite profiles. As illustrated in Figure 3.1a, the fermentation process could be divided into two main stages by glucose depletion. At the beginning of first stage, when ORP had first dropped to -260 mV at the 7^{th} h, the cell had entered the exponential phase from lag phase based on OD_{600} reading. From this point and forward, the specific glucose utilization rate increased. Lactate, ethanol and acetate were the major products as glucose was largely consumed. The glucose depletion was reflected as the lowest level (-280 mV) in ORP profile around 28^{th} h. Meanwhile, the cell had entered the stationary phase as OD_{600} reached its maximum reading at 8.1. In the second stage, glycerol was utilized as the maintenance carbon source for cell survival while producing PDO. The glycerol utilization and PDO production rates were found to be the highest when ORP was fluctuated around -250 mV. Furthermore, during the glycerol utilization in the stationary phase, the DO level (0.2657 ± 0.025 mg/L) was nearly constant (data not shown). Lactate was consumed with glycerol, yielding PDO and acetate, while ethanol was nearly invariable after glucose was exhausted. Throughout the entire course of fermentation, ORP was oscillated between -240 to -280 mV between 7^{th} h and 41^{st} h until ORP continuously increased. The final PDO production was 237.21 mM (18.05 g/L) after 104.2 h without ORP control.

As seen in Figure 3.2a, rapid uptake of glycerol and lactate along with the production of acetate and PDO became noticeable, implying that glycerol could utilize NADH faster than ethanol. Figure 3.2a also shows that most acetyl-phosphate was converted by acetate kinase to acetate rather than ethanol, so that one extra ATP was gained [10],[27]-[28]. A portion of pyruvate was converted by pyruvate oxidase, becoming acetyl-phosphate instead of lactate. After the exhaustion of glucose, the pathway from pyruvate to lactate had been

reversed, accompanying with NADH regeneration to support cell survival and glycerol reductive pathway. The NADH generated was then used for PDO production (Figure 3.2a). Glycerol was mainly consumed after glucose had been depleted under no ORP-controlled condition, and the consumption of glycerol was coupled with lactate utilization to maintain redox balance. The gene responsible for glycerol metabolism in *Lactobacilli* are encoded in *pdu* operon, where Coenzyme B12-dependent glycerol dehydratase (GDHt), encoded by *dhaB*, converts glycerol to 3-hydroxypropanaldehyde (3-HPA), and then is further reduced by the NADH dependent enzyme 1,3-propanediol dehydrogenase (PDOR), encoded by *dhaT*, to produce PDO [5],[7]-[10].

3.4.2 Redox Potential Control and the Timing of Supplementing Potassium Ferricyanide

Since ORP fluctuated around -250 mV under no ORP-controlled condition, the subsequent experiments were designed to control ORP at -250, -200 and -150 mV, respectively. Potassium ferricyanide, an inorganic-based oxidant, features a high reduction potential (436 mV) was used in this study. The timing to trigger the oxidant supplementation was based on the pre-determined ORP setpoints. When ORP level was below the setpoint, the oxidant was supplemented. As shown in Figure 3.1b-d, the arrows indicate the initiation of supplementation of potassium ferricyanide.

By correlating the biomass profile to the ORP profile, Figure 3.1b –d have also illustrated that the moment when the oxidant was added fallen within the exponential growth phase of PM1 for all three ORP-controlled cases. The ORP regulation spanned from the early exponential phase to the late exponential phase, creating a relatively oxidized environment (with respect to no ORP control counterpart) to favor glycerol reductive pathway for PDO synthesis.

The addition of potassium ferricyanide had significantly shortened fermentation time (Table 1). Controlling ORP at -250 mV started at almost the end of exponential phase (Figure 3.1b) where there was the least amount of glucose available (Figure 3.2b). ORP was maintained at -250 mV for about 3.5 h and the remining glucose was completely consumed until ORP raised up. The final PDO produced was 245.07 mM (18.62 g/L). The experiment was further carried out to control ORP at -200 mV (Figure 3.1c), where an even more oxidized environment stimulated glycerol and glucose consumption, leading to the highest PDO production of 304.57 mM (23.38 g/L, Figure 3.2c). However, when ORP was controlled at -150 mV (Figure 3.1d), the cell had not built up enough population to completely exhaust glucose (OD_{600} = 4.02 as compared to 4.41 under -200 mV, and 5.69 under -250 mV), resulting in lower PDO concentration of 260.91 mM (19.76 g/L) and longer control period (9 h compared to 3.5 h when controlling ORP at -200 mV). Hence, Controlling ORP at -200 mV could be regarded as the most favorable setting under the current investigation. It can be seen that the duration of ORP regulation was dependent on the residual glucose concentration. Among all the controlled cases, the higher the ORP control setpoint, the higher the residual glucose was, consequently longer ORP control period was required. The end of ORP control period was coincided with the glucose de-

pletion, creating a relatively oxidized environment for further glycerol reduction. Combining Figures 3.1 and 3.2, it became apparent that ORP regulation could trigger and accelerate glycerol utilization. Controlling ORP at -200 mV which was equivalent to supplementing potassium ferricyanide near the mid-exponential growth phase, would result in the most productive fermentation outcome in terms of final PDO concentration and batch fermentation period (Table 1).

3.4.3 Effect of Potassium Ferricyanide on the Metabolite Profiles

When PM1 was grown under no ORP-controlled condition, glycerol consumption was activated after complete depletion of glucose, at which the corresponding growth stage for PM1 was in the stationary phase, and the lactate was at its highest concentration. Once activated, the utilization of glycerol was accompanied by the consumption of lactate (Figure 3.2a). As fermentation proceeded, lactate served as electrons donor, contributing two electrons for NADH regeneration, which subsequently incorporating into PDO synthesis along with the consumption of glycerol. As a result, the NAD^+/NADH balance could be maintained. Pyruvate, generated through the consumption of lactate, was mainly used for acetate synthesis, where one mole of ATP was generated. Note that the change of ethanol concentration after the glucose exhaustion was insignificant. This indicated that the major NAD^+ contributor to facilitate PDO production was coming from the utilization of lactate rather than from the consumption of ethanol (Figure 3.2, Figure 3.3).

When ORP control was implemented through the addition of potassium ferricyanide, the glucose consumption rate, the glycerol consumption rate, the lactate consumption rate, and the PDO production were increased; particularly, for the case of controlling ORP at -200 mV (Figure 3.3). The highest glucose and glycerol consumption rate and PDO production rate occurred when glucose was nearly depleted at the end of ORP regulation.

During ORP regulation, supplementing potassium ferricyanide as an oxidizing agent reshuffled NAD^+/NADH recycling from glycolysis to glycerol reductive pathway, leading to higher glycerol utilization and PDO production rates. Two moles of NADH supposed to be utilized in ethanol synthesis had been shifted into PDO production. Note that ethanol concentration remained nearly constant after the complete depletion of glucose. On the other hand, the continually build up of acetate indicated that the metabolic route from pyruvate, acetyl-CoA to acetyl-phosphate was not affected by the addition of potassium ferricyanide. The above observations about the fate of acetyl-phosphate and ethanol were the same as that for no ORP-controlled case.

Although similar fate of ethanol profile under no ORP and ORP-controlled environment, the final ethanol concentration under ORP regulation was reduced at least in half of that without the supply of oxidant (Table 3.1). This implied that the acetaldehyde dehydrogenase and alcohol dehydrogenase governing the conversion of acetyl-CoA to ethanol was somehow under suppression. Table 3.1 summaries that the final acetate concentration obtained under no ORP or with ORP-controlled conditions resulted in negligible difference, and the final concentration ratio of PDO to acetate was almost constant (1.99–2.05) irrespective of the presence

or absence of potassium ferricyanide.

When the growth of PM1 was subjected to ORP control, the presence of potassium ferricyanide did not alter the way that lactate was being synthesized as the formation of lactate was tied to the glucose utilization. Lactate would continue to be synthesized until the depletion of glucose. Most importantly, lactate, as NAD^+ donor, activated glycerol reductive pathway, resulting in the production of PDO.

3.4.4 Productivity of 1,3-Propanediol

The previous studies on the PDO production from glycerol by heterofermentative *Lactobacillus* are summarized in Table 3.2. The highest PDO production was 92 g/L by *L. diolivorans* DSM14421 cultivated in fed-batch fermentation [28]. The highest PDO productivity was achieved by *L. reuteri* CH53 grown under fed-batch environment, with 1.27 g/L/h and the final PDO concentration was 68 g/L [29]. Heterofermentative *Lactobacillus* converts glycerol to PDO, with glucose as the reducing substrate to support cell growth. The PDO production is closely related to the availability of biomass, in which the synthesis of PDO is mainly happened in the stationary phase. In this study, the incorporation of ORP regulation to batch fermentation has significantly improved the fermentation efficiency in terms of shortening fermentation time and increasing PDO production.

ORP controlled at -200 mV in this study had resulted in the highest PDO mass yield of 0.82 with respect to glycerol under batch fermentation mode. The highest PDO productivity was 0.48 g/L/h when controlled ORP at -200 mV. This reported PDO yield was even higher than those obtained using fed-batch fermentation (Table 3.2).

3.5 Conclusions

Incorporating redox potential control to fermentation has resulted in the enhancement of PDO production and the reduction of batch fermentation time. Without ORP control, PM1 consumed glucose and glycerol in sequential order. When ORP was implemented, the co-substrate utilization pattern was observed. However, the timing of supplementing potassium ferricyanide (*ie,* ORP control level) was crucial as it would affect the PDO production. The best ORP control level in this study was -200 mV, which was equivalent to the mid-exponential growth phase of PM1. It is postulated that the regulation of fermentation redox potential using potassium ferricyanide altered NAD^+/NADH balance by reshuffling electrons used by reducing equivalent pairs to favor PDO production. Consequently, there were unnoticeable changes in ethanol concentration when ORP control was initiated. Although the obtained PDO yield was the highest among all compared studies, but the final PDO concentration was low. It is mostly because of the glycerol concentration used in this study was the lowest. Considering yield is 0.82, higher glycerol concentrations and/or feeding glycerol during the stationary phase could increase the PDO production further. Note that PM1 used in this study was not specifically isolated for PDO production as those strains cited in Table 3.2. Instead, it is the wild-type

strain isolated from thin stillage of a local bioethanol plant during bioethanol production. We extrapolate that if this demonstrated ORP regulation technology were implemented to those *Lactobacilli* as mentioned here, their PDO productivity would be further improved.

3.6 Acknowledgement

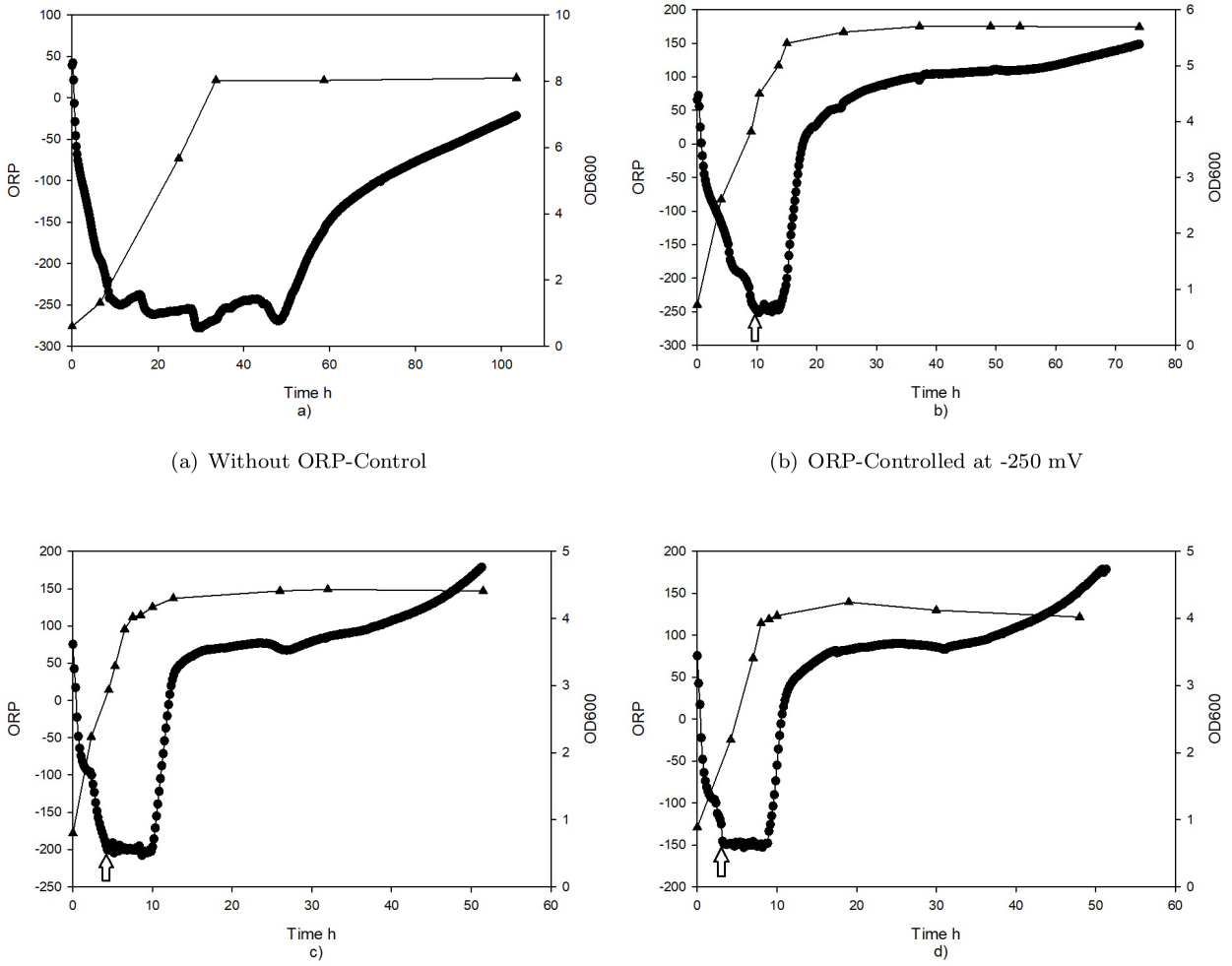
The authors acknowledge the financial support from the Natural Sciences and Engineering Research Council of Canada.

Table 3.1: Effect of ORP-Controlled PM1 fermentation from 21.5 g/L Glucose and 33.0 g/L Glycerol

	No ORP Control	ORP Controlled at -250 mV	ORP Controlled at -200 mV	ORP Controlled at -150 mV
Fermentation Time h	104	74	49	49
Final OD_{600}	8.12	5.69	4.41	4.02
Time of Glucose Depletion h	28	13.2	10.5	12
Glycerol Residual mM	63.54±0.25 (5.85 g/L)	67.54±0.26 (6.22 g/L)	45.94±0.18 (4.23 g/L)	56.36±0.22 (5.19 g/L)
Lactate mM	26.92±0.46 (2.42 g/L)	80.40±0.38 (7.24 g/L)	79.08±0.40 (7.12 g/L)	64.26±0.37 (5.78 g/L)
Acetate mM	133.57±0.21 (8.02 g/L)	121.96±0.18 (7.32 g/L)	148.82±0.15 (8.94 g/L)	126.17±0.20 (7.58 g/L)
Ethanol mM	106.02±0.24 (4.88 g/L)	61.19±0.31 (2.82 g/L)	36.54±0.28 (1.68 g/L)	43.70±0.35 (2.01 g/L)
PDO mM	237.21±0.12 (18.01 g/L)	245.07±0.13 (18.62 g/L)	304.57±0.09 (23.38 g/L)	260.91±0.12 (19.76 g/L)
PDO/Glycerol mass yield g/g	0.66	0.70	0.82	0.71
PDO productivity g/L-h	0.17	0.25	0.48	0.40

Table 3.2: PDO Fermentation from Glycerol by Heterofermentative *Lactobacillus*

Strains	Fermentation Strategy	PDO g/L	PDO Productivity g/L/h	Approximate Fermentation Time h	Yield g/g PDO/Glycerol	Reference
L. reuteri ATCC55730	Batch	37.4	0.15	249	0.59	[27]
L. reuteri DSM20016	Batch	46	0.66	70	0.74	[17]
L. diolivorans DSM14421	Batch	41.7	0.30	139	0.65	[28]
PM1	Batch	23.38	0.48	49	0.82	This Study
L. reuteri ATCC55730	Fed-Batch	65.3	0.47	139	0.80	[27]
L. diolivorans DSM14421	Fed-Batch	85	0.45	189	0.47	[30]
L. diolivorans DSM14421	Fed-Batch	92	0.56	165	0.78	[16]
L. reuteri CH53	Fed-Batch	68	1.27	54	0.82	[29]



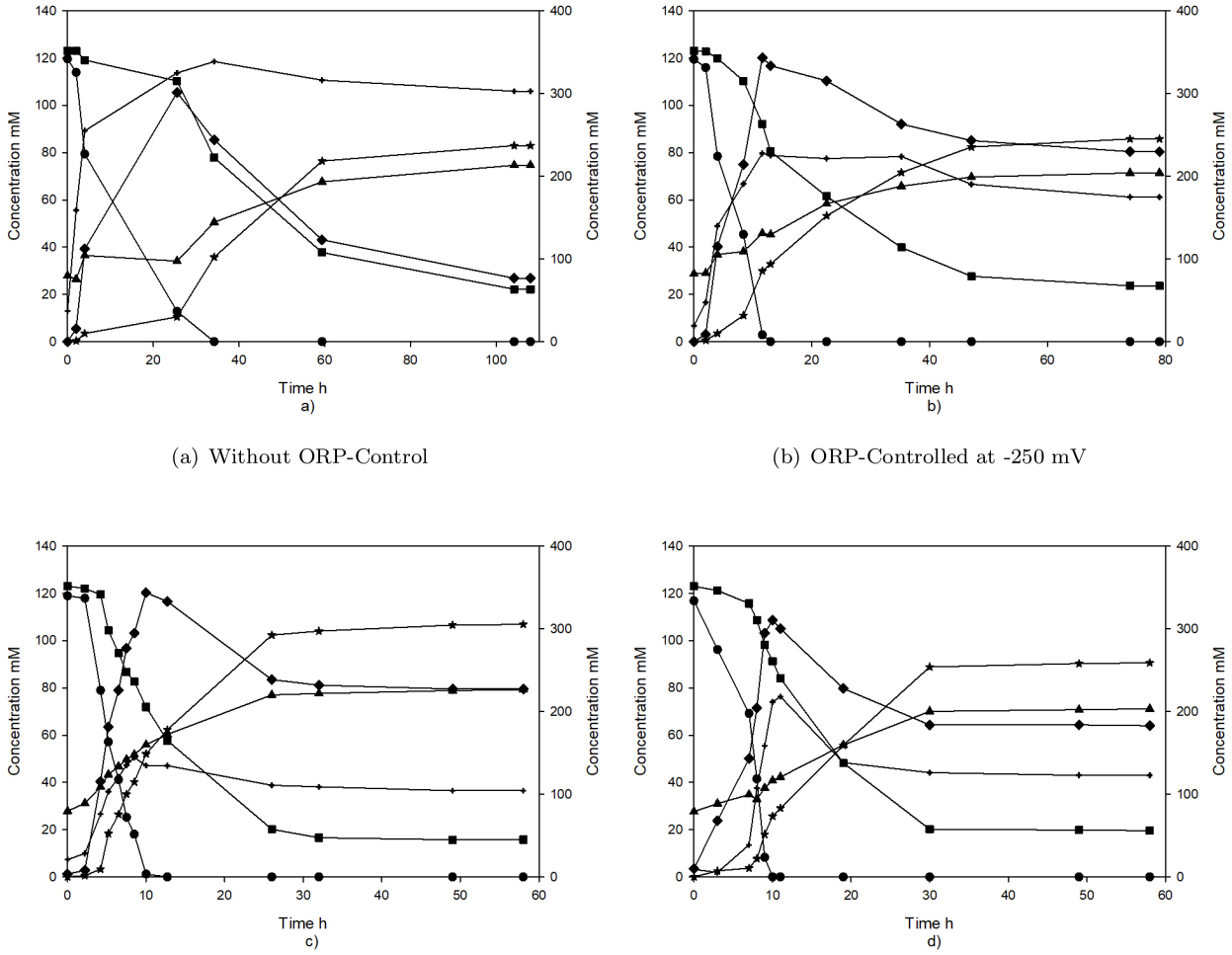
(a) Without ORP-Control

(b) ORP-Controlled at -250 mV

(c) ORP-Controlled at -200 mV

(d) ORP-Controlled at -150 mV

Figure 3.1: PM1 Profiles in ORP (circle) and OD_{600} (triangle)



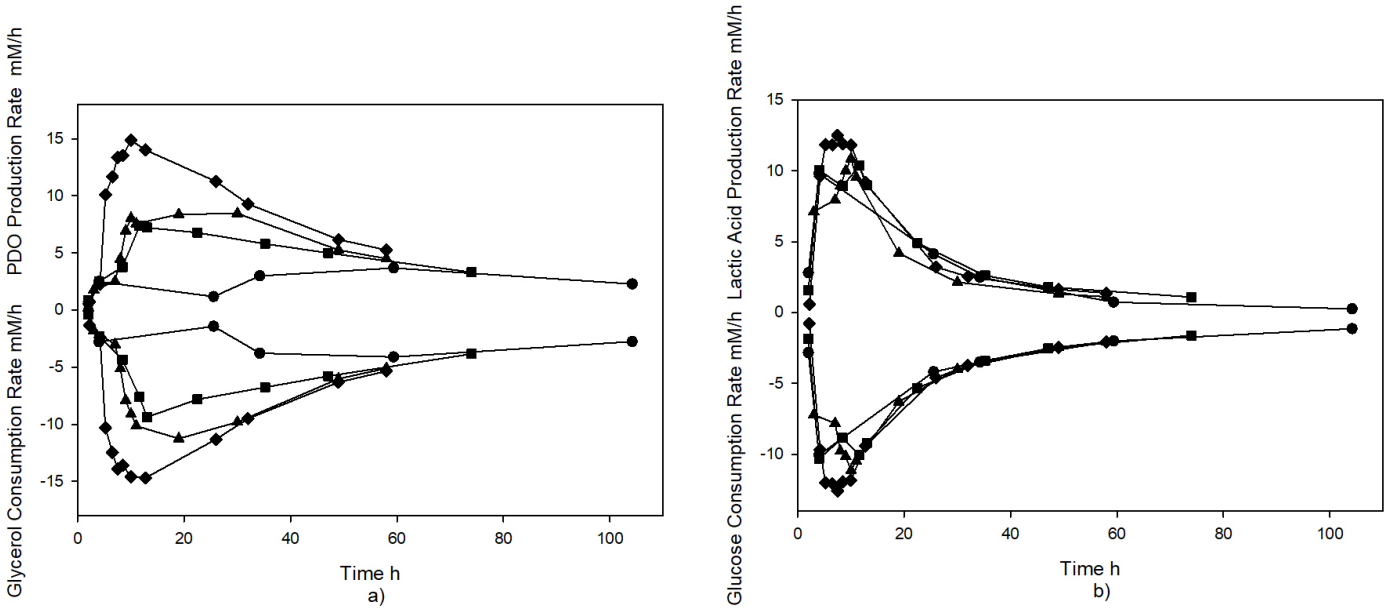
(a) Without ORP-Control

(b) ORP-Controlled at -250 mV

(c) ORP-Controlled at -200 mV

(d) ORP-Controlled at -150 mV

Figure 3.2: Metabolic Profiles of PM1 in Glucose (circle, left); Lactate (diamond, left); Ethanol (plus, left); Glycerol (square, right); Acetate (triangle, right); PDO (star, right)



(a) Glycerol Consumption and PDO Production Rate

(b) Glucose Consumption and Lactate Production Rate

Figure 3.3: PM1 Substrates Consumption and Product Production Rate with ORP Regulation at -250 (square), -200 (diamond), -150 (triangle) mV and without ORP Control (circle)

4

Conclusions and Recommendations

4.1 Summary of Results

For all PDO fermentations by PM1 with 21.5 g/L glucose and 33.0 g/L glycerol, the temperature was maintained at 37℃, pH was regulated at 5.5 and agitation was controlled at 100 rpm.

Without ORP control, the fermentation lasted for 104 h, with final OD$_{600}$ of 8.12. The final PDO production was 18.01 g/L with productivity of 0.17 g/L/h. The residual glycerol was 5.85 g/L, along with 2.42 g/L lactate, 8.02 g/L acetate and 4.88 g/L ethanol produced.

For ORP controlled at -250 mV, the fermentation lasted for 74 h, with final OD$_{600}$ of 5.69. The final PDO production was 18.62 g/L with productivity of 0.25 g/L/h. The residual glycerol was 6.22 g/L, along with 7.24 g/L lactate, 7.32 g/L acetate and 2.82 g/L ethanol produced.

For ORP controlled at -200 mV, the fermentation lasted for 32 h, with final OD$_{600}$ of 4.41. The final PDO production was 23.38 g/L with productivity of 0.48 g/L/h. The residual glycerol was 4.23 g/L, along with 7.14 g/L lactate, 8.94 g/L acetate and 1.68 g/L ethanol produced.

For ORP controlled at -150 mV, the fermentation lasted for 40 h, with final OD$_{600}$ of 4.02. The final PDO production was 19.76 g/L with productivity of 0.40 g/L/h. The residual glycerol was 5.19 g/L, along with 5.78 g/L lactate, 7.58 g/L acetate and 2.01 g/L ethanol produced.

The glucose and glycerol consumption rates under ORP regulation were at least 2-5 times higher than those without regulation. The addition of potassium ferricyanide stimulated the glycerol consumption with glucose from the middle of exponential phase, resulting in higher PDO production and lower lactate consumption after glucose depletion.

The most optimal molar ratio between glucose to glycerol was evaluated among 0.14, 0.24, 0.34 and 0.44 under -200 mV. Based on the results shown in Table 4.1, the most optimized R value is ranged between 0.30-0.40. For R value falled below this range, there was insufficient glucose to support the glycerol reduction during ORP regulation. Increasing the R value, the resulting high glucose concentration did not have any significant effect on PDO production. Instead, it prolonged the ORP regulation period since there were more glucose needed to be consumed. Based on ORP regulation period and the final PDO concentration, maintaining R value between 0.30 to 0.40 was regarded as the best ratio in this study.

Table 4.1: PDO Production from Different Initial Molar Ratio (R) of Glucose to Glycerol (35.92 g/L) with ORP-controlled at -200 mV.

R	0.14	0.24	0.34	0.44
ORP Regulation Time h	1.72	2.85	4.41	6.12
PDO Production mM	84.18 (6.40 g/L)	258.25 (19.65 g/L)	304.57 (23.38 g/L)	298.27 (22.70 g/L)

4.2 Conclusions

ORP regulation has significantly lowered the fermentation time by initiating glycerol consumption with glucose in the exponential phase, resulting in the improvement of PDO production. When PM1 was grown without ORP control, glycerol consumption was started after glucose depletion at the stationary phase. The utilization of glycerol was accompanied by the consumption of lactate produced from glycolysis. After the exhaustion of glucose, lactate utilization mode switched to consumption to restore the NAD^+/NADH balance and cellular maintenance. The addition of oxidizing agent shifted the metabolic pathway from glycolysis to glycerol reduction, leading to higher glycerol consumption and PDO production rate. The higher the ORP control setpoint, the lower the biomass was. ORP controlled at -200 mV, it resulted in the highest PDO production (23.38 g/L), and the shortest batch fermentation time.

4.3 Recommendations and Future Work

4.3.1 ORP controlled Fed batch Fermentation

PDO production and fermentation efficiency could be further improved through ORP-controlled fed-batch fermentation. In this study, there were glycerol residual reminded after the end of the fermentation (5.85 g/L without control; 6.22 g/L at -250 mV; 4.23 g/L at -200 mV; 5.19 g/L at -150 mV). ORP controlled at -200 mV with the lowest glycerol residual has suggested the feasibility of ORP-controlled fed-batch fermentation in glycerol residual elimination for future study. Based on previous study, fed-batch fermentation resulted in higher PDO production by *Lactobacillus*. The substrate concentration can be modified to support strain growth and metabolism. *L. reuteri* produced up to 65.3 g/L PDO under fed batch fermentation at pH 5.5, 37℃ with initial 20 g/L glucose and 10 g/L glycerol. A feed with molar ratio glucose to glycerol of 1.5 was supplied after complete consumption of glucose [27]. *L. diolivorans* produced up to 84.5 g/L PDO in fed-batch fermentation with co-feeding glucose and glycerol in molar ratio at 0.1. This experiment was carried out under pH 5.5, 37℃ with initial 30 g/L glucose and 10 g/L glycerol [28]. If ORP controlled fed-batch PDO fermentation is applied, another ORP control level for glycerol reduction need to be defined, to provide suitable reduction environment to maintain glycerol reduction to PDO after batch fermentation. For PM1,

supplementing glucose after the elimination of initial glucose in batch fermentation, does not improve the PDO production since PDO is only converted from glycerol [10]. The initial substrates concentration, feed ratio and feed rate need to be evaluated to improve PDO production and to eliminate residual glycerol.

4.3.2 Redox Balance Genes Activities under Different ORP Environment by RT-PCR

Since the addition of potassium ferricyanide shifted the PM1 metabolism. The gene activities relateing to redox sensitive enzymes should be investigated under different ORP regulation levels. These genes are alcohol dehydrogenase and acetaldehyde dehydrogenase, involved in acetyl-CoA to ethanol; acetate kinase, involved in acetyl-phosphate to acetate and ATP production; glyceraldehyde 3-phosphate dehydrogenase, involved in glyceraldehyde 3-phosphate to 3-phosphate glycerate; lactate dehydrogenase, involved in pyruvate to lactate; pyruvate dehydrogenase, involved in pyruvate to acetyl CoA; phosphotransacetylase, involved in acetyl-phosphate to acetyl-CoA. The activities of these genes can be tested by RT-PCR for comparative CT experiment, where 16S RNA is the endogenous control [7].

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Appendix A

Experimental Setup

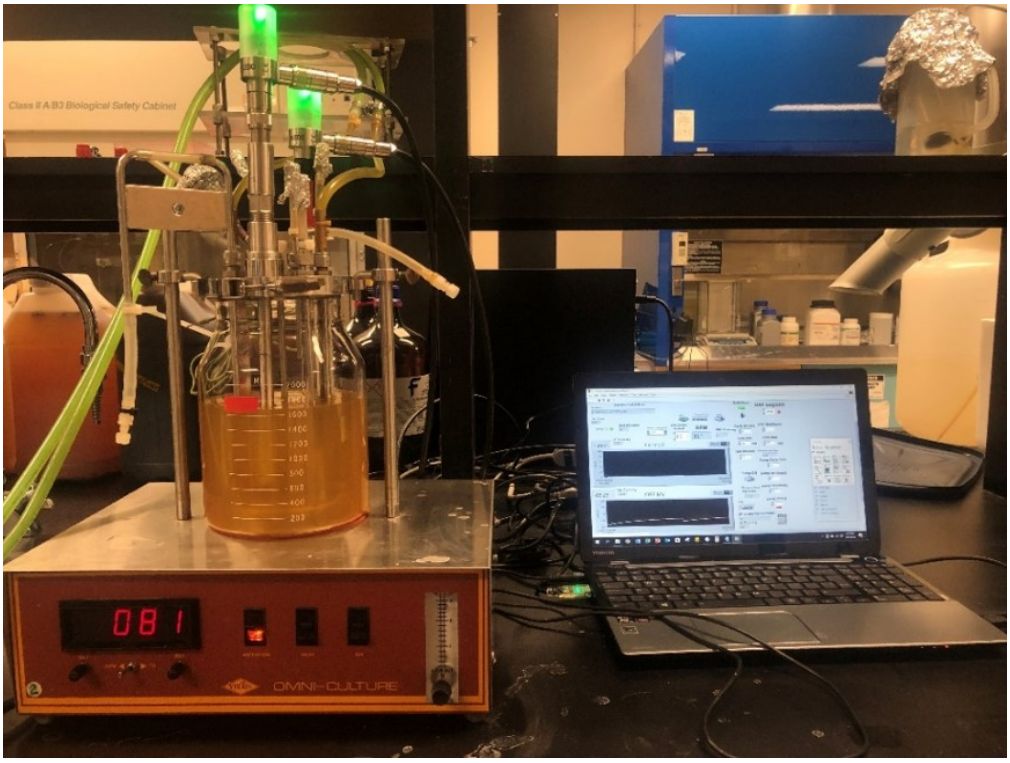

Figure A.1: Experimental Setup

Figure A.2: PM1 Fermentation after 36 hours

Appendix B

PM1 Inoculation

All procedures of inoculation below are performed in the biosafety cabinet.

B.1 Preparation of Modified MRS Broth

1. Weigh out 21.5 g glucose, 5 g yeast extract, 10 g peptone, 10 g meat extract, 2 g K_2HPO_4, 2 g ammonia citrate, 5 g sodium acetate, 100 mg $MgSO_4 \cdot 7H_2O$, 50 mg $MnSO_4$ and 33.0 g glycerol. Dissolve the powder in 1000 mL of distilled water, and divide the media equally into two 1-L autoclavable bottles.
2. Loose the lid to the bottle, and cover the lid with aluminium foil. Label the bottles by autoclave tape with: media name, date, lab number, and initials.
3. Autoclave the samples using Liquid 15 cycle.

B.2 Preparation of Modified MRS Agar Plate

1. Weigh out 7.5 g of agar and other chemical mentioned above. Dissolve the powder in 1000 mL of distilled water, and divide the media equally into two 1-L autoclavable bottles.
2. Loose the lid to the bottle, and cover the lid with aluminium foil. Label the bottles by autoclave tape with: media name, date, lab number, and initials.
3. Autoclave the samples using Liquid 15 cycle.
4. Cool the agar down in a 75˚C water bath.
5. In the biosafety cabinet, pour agar into petri plates covering approximately 3/4 of the plate. Close the lids to the plates and swirl gently to distribute the agar across the petri plate.
6. Allow the plates to solidify. Store them at 4˚C.

B.3 Preparation of PM1 after Receiving from -80°C Stock

1. In the biosafety cabinet, open the vial and aseptically transfer the glycerol stock to tubes containing modified MRS broth (5 to 6 mL).
2. Mix contents of the tubes by pipetting up and down gently.
3. Using a sterile loop, streak the culture into modified MRS agar plates.
4. Incubate the tubes and plates at 37˚C for 24-48 hours.

B.4 PM1 Inoculation (Plate to Plate)

1. Use sterile loop to attach single colony of PM1 from original agar plate. Close the original petri dish.
2. Hold the charged loop parallel with the surface of the agar, smear the inoculum backwards and forwards across a small area of the medium.
3. Inoculate the agar plate at 37˚C incubator until colonies shown up.
4. Seal and store at 4˚C for maximum two weeks.

B.5 PM1 Inoculation (Plate to Broth)

1. Use sterile loop to attach single colony of PM1 from agar plate.
2. Dip the loop inside a 10 ml Falcon tube containing 5 mL of liquid modified MRS medium and mix slowly.
3. Incubate the culture medium at 37°C for 12 to 20 hours.

B.6 Optical Density Determination

(The OD reading can be performed on the bench)
1. Take 1 mL of culture and put it into a plastic cuvette.
2. Clean the outside of the cuvette with a kimwipe to remove contamination interfereed with the OD reading.
3. Use UV-Vis spectrophotometer to measure the absorbance of 1 ml incubated MRS medium at 600 nm (MRS culture as blank). If the OD is greater than 0.6, the sample dilution is needed.

Appendix C

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